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(54) Title: ANTIFREEZE GLYCOPEPTIDE COMPOSITIONS TO PROTECT CELLS AND TISSUES DURING FREEZING

(57) Abstract

The present invention relates to aqueous compositions of substances, such as organic molecules, which are useful to protect and preserve viable plant or animal cell membrane and tissue exposed to hypothermal and hyperthermal temperatures or non-physiological chemical conditions, and to modify the freezing process of liquids in biological plant or animal cells or tissue. More specifically, the present invention relates to the use of antifreeze polypeptide or antifreeze glycopeptide which is derived, for example, from the fluid or serum of Artic and Antarctic fish. Preferred antifreeze compounds are related to those polypeptides having multiple alanine-alanine-threonine- or alanine-alanine-alanine- segments. In some embodiments, a pendant sugar group is covalently attached to each threonine moiety. An aqueous solution of the peptide or glycopeptide is contacted with cells ova, sperm, oocytes, embryos, tissue, an organ, or a whole living plant or animal. The cells, tissue, organ or plant or animal is then carefully cooled and/or frozen at 0 °C or below (in some cases to -196 °C or to 4 K) and held at the low freezing (or vitrification) temperatures. The ice forms, if at all primarily along the c-axis with the result that cell membranes are not disrupted and the cells are not dehydrated. The cells, tissue, whole plant or organ or animal are carefully thawed, and all are found to be viable. The preserved organs are particularly useful transplant organs for a human being.

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ANTIFREEZE GLYCOPEPTIDE COMPOSITIONS TO PROTECT CELLS AND TISSUES DURING FREEZING

Field of the Invention

The present invention relates to aqueous compositions of substances, e.g. thermal hysteresis protein, which are useful to modify the freezing process of liquids in biological tissue. More specifically, the present invention relates to the use of antifreeze protein or glycoprotein which is derived, for example, from the fluid or serum of Arctic and Antarctic fish. Preferred antifreeze compounds are related to those obtained from natural animal sources. More preferred are those polypeptides having multiple -alanine-alanine-threonine- or -alanine-alanine-alanine- segments. In some embodiments, a pendant sugar group is covalently attached to the threonine moiety.

A solution of antifreeze protein is perfused through animal tissue or an animal organ. The tissue or organ is then carefully frozen to temperatures below -0.5°C and held at the low temperatures. The ice forms primarily along the c-axis of the ice crystal, and ice formation is inhibited in the direction of the a-axes (faces) of the ice crystal. This spicular ice growth compartmentalizes the concentration of the salts with the result that adjacent cells are not disrupted or completely dehydrated. The tissue or organ is carefully thawed, and the tissue or organ is functioning and viable. The preserved organs are particularly useful in transplantation therapy in a human being.

The present invention also relates to compositions of substances, e.g. thermal hysteresis protein which are useful to improve survival, functionality and/or structural integrity in biological materials, e.g., microorganisms, animal cells, tissues or organs exposed to temperatures and chemical environments different from their normal physiological temperatures and environments, by protecting at least the cell membranes from damage and cell contents from leakage due to exposure to nonphysiological thermal and

chemical conditions.

DESCRIPTION OF RELATED ART

The preservation of viable animal tissue, animal organs and living animals has been the subject of recent intense laboratory and medical research. Human organ transplants of heart, kidney, lung, liver and the like are now possible because of improved surgical techniques, improved anti-rejection drugs, and immediate availability of donated organs. Presently, donor organs are removed from a donor, cooled, stored on wet ice, but not frozen and within a maximum of a few hours are surgically placed in a recipient's body.

The preservation of animal tissue, animal organs and intact viable animals by freezing at lowered temperatures is presently limited to a few hours, because the normal formation of ice in an organ produces localized concentrated salt solutions. Water migrates from the nearby cells irreversibly dehydrating the cell. These events are major problems that disrupt the organ structure and function, and the organ does not reactivate or function upon thawing.

Advances in the development of immunosuppressants, improvements in organ transplantation techniques and the successful use of freezing for long-term preservation of cells have motivated intensive research efforts on methods for long-term preservation of biological organs through freezing. Recently, B. Rubinsky, U.S. Patent 4,531,373 disclosed an experimental technique using a directional solidification stage and low temperature scanning electron microscopy to facilitate the study the process of freezing in biological tissues.

B. Rubinsky et al., (1988) Proceedings of the Royal Society London, B., Vol. 234, pp. 343-358), also describes experimental results and a mathematical model for the freezing process and the mechanism of damage in biological tissue and biological organs.

None of the available literature below disclose a composition or a method to preserve for long times tissue or

organs.

Earlier experimental results show that single, continuous ice crystals normally form along the blood vessels of frozen tissue. B. Rubinsky et al. (1988), Cryo-Letters, Vol. 8, p. 370; B. Rubinsky et al. (1988), Proc. Royal Soc. Lond., B234, 343. The structure of the frozen tissue depends on the cooling rate, (i.e., the temperature variation per unit time) during freezing. When tissue, such as liver, is frozen with low cooling rates (about 1°C/min to about 10°C/min), the smaller blood vessels (sinusoids) expand relative to those of the unfrozen normal liver tissue. In addition, the cells (hepatocytes) adjacent to the expanded sinusoids, are dehydrated without intracellular ice forming. However, at higher cooling rates, intracellular ice forms in the cells (hepatocytes) resulting in a reduced expansion of the sinusoids.

One explanation for the observed formation of continuous ice crystals along the blood vessels, for the expansion of the frozen blood vessels, and for the formation of intracellular ice during freezing with higher cooling rates is that ice formed in the vascular system does not propagate through the cell membranes or the blood vessel wall. Instead, ice forms within and propagates along the blood vessels where there is no barrier to the ice crystal growth process. Water in the cells surrounding the frozen blood vessels, being compartmentalized in small volumes, will, at first, remain supercooled. As the intravascular ice forms, water is removed from the solution in the vascular space, rendering the remaining solution hypertonic (higher in salts concentration). This higher concentration of solutes causes water to migrate irreversibly from the surrounding cells, through the semi-permeable cell membrane, into the blood vessel in order to equilibrate the difference in chemical potential. Consequently, the cells surrounding the blood vessel will dehydrate, and the water that leaves the cell then freezes in the vascular system. Water transport from cells through the cell membrane into the

blood vessel, is a rate-governed process, which depends on the permeability of the cell membrane. Therefore, when larger organs are frozen using higher (i.e. faster) cooling rates, sufficient water remains in the cell for intracellular ice to form prior to the complete dehydration of the cell. A more detailed description of the process of freezing and a mathematical model that supports this description is found in the Rubinsky, et al. (1988) reference above. This result also leads to the conclusion that one of the possible modes of damage to frozen tissue is the observed expansion of the blood vessels which causes the disruption of the structural (mechanical) integrity of the organ. This mode of damage apparently does not affect cells frozen in suspensions, and may explain why organs do not survive freezing under the same conditions in which cells in suspensions survive.

The normal patterns of ice formation, in which the energetically preferred direction of ice growth is also the a-axes (prism face) of the hexagonal prism ice crystal, governs the process of freezing in tissue. Any hexagonal prism facet of the a-axes of the three-dimensional ice crystal has the same energetic preference and, therefore, during freezing of tissue, the ice crystal can continuously follow and grow along the blood vessel. Furthermore, as discussed earlier, the large ice crystals of normal freezing do not incorporate solutes. This rejection of solutes results in more concentrated solutes, a mass transfer process and the irreversible water migration from local cells and tissue into the open vessel. This migration leads to the disruption of the structural integrity of the cells of the tissue or organ.

Additional background information can be found in:

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All references, patents, patent applications, articles, standards, etc. cited in this application are incorporated herein by reference in their entirety.

It would be extremely advantageous to have a composition and a method which would alter the preservation process of biological liquids in animal cell tissue. Thus, when the frozen tissue, organ, plant or animal is carefully thawed, it results in viable cells, tissue, organ, plant or animal. The present invention provides such a preservation composition and method.

SUMMARY OF THE INVENTION

The present invention in one aspect relates to a composition useful in the protection and preservation of viable cells and cell membranes of an animal independently subjected to :

(i) hypothermal temperature conditions from the physiological temperature to about 0°C,

(ii) vitrification temperature conditions from about 0°C to about -190°C,

(iii)freezing temperatures from between about -0.5°C and 4K,

(iv) hyperthermal temperature conditions from the physiological temperature up to about 10°C above the physiological temperature, or

(v) nonphysiological chemical conditions, or

(vi) or combinations thereof which composition comprises:

one or more thermal hysteresis proteins; and

a biologically compatible aqueous preservation solution.

The present invention is related to a composition of biologically compatible substances, e.g., antifreeze peptide, useful to the survival, functionality, stability and structural integrity of biological materials, including proteins, enzymes, lipids, cell membranes, animal or plant cells, microorganisms, tissues, organs, whole animals, or whole plants subjected to nonphysiological temperatures, either higher or lower than the normal physiological temperatures or to nonphysiological chemical environments, by interacting with the proteins, lipids and at least the cell membranes.

In other aspects, the present invention also relates to a composition of antifreeze protein useful in improving survival, functionality, stability and structural integrity of biological materials, including animal or plant proteins, enzymes, lipids, carbohydrates, cell membranes, cells (animal or plant) microorganisms, tissues or organs subjected to temperatures lower physiological and lower than 0°C in the presence of ice crystals:

(a) by modification of the structure of the ice crystals in contact with the proteins, enzymes, lipid or at least the plant or animal cell membranes;

(b) by reducing the number and the size of the ice crystals or by completely eliminating the ice crystals in contact with the proteins, enzymes, lipids or at least the cell membranes; or

(c) by modifying the mode in which solutes are rejected by the ice formation and thereby changing the chemical composition of the solution surrounding the proteins, enzymes, lipids or at least the cell membranes.

The present invention also relates to a composition useful to block ion channels in membranes to retard or prevent ion leakage (in general) and to stabilize cell membranes (in general), or in binding other macromolecules

to proteins, lipids, or at least cell membranes.

The composition whose usefulness discussed above comprises at least one biologically compatible antifreeze substance, and a biologically compatible aqueous solution.

In one aspect the biologically compatible antifreeze substance is a macromolecule (e.g. a polypeptide) from or substantially the same as a macromolecule derived obtained from an animal selected from fish, amphibian, worm, insect or reptile, preferably fish from Arctic, Antarctic, North Temperate or South Temperate Zones. More preferably, the protein is from body fluids (e.g. blood) from Antarctic fish, e.g. from the family Nototheniidae, including the species D. nawsoni and P. borchgrevinki or the Antarctic eel pout Rhigophila dearborni, or the Arctic winter flounder. All these antifreeze proteins are known and have the common property that they modify the structure of ice crystals.

In one embodiment, the biologically acceptable substance is selected from a polypeptide, a glycopeptide, a polypeptide covalently bonded to biologically acceptable carrier, a glycopolypeptide covalently bonded to a carrier or mixtures thereof.

In one embodiment, the aqueous composition further includes additional preserving, protecting or vitrifying compounds selected from glycerol, dimethylsulfoxide, ethylene glycol, polyvinylpyrrolidone, glucose, sucrose, propanediol, propylene, glycol, carboxymethyl cellulose, or mixtures of these compounds which are known to protect cells and biological materials against freezing damage or to promote vitrification.

The ability of the compounds to protect or stabilize membranes are also useful in the preservation of food; in cosmetics to restore, preserve or repair skin tissue; or in therapy for diseases associated with instability of cell membranes.

The ability to block ion channels is used in treating diseases associated with imbalances of the intracellular-extracellular ion transport across cell membranes.

The ability to attach to and interact with cell membrane, is used in attaching various macromolecules to the antifreeze proteins and thereby facilitating their attachment to the cell membrane.

In another aspect the present invention relates to a method for preservation, survival, functionality, stability and structure or integrity of biological materials, at non-physiological temperatures or in nonphysiological chemical compositions, including proteins, enzymes, lipids, cell membranes, cells (animal or plant), microorganisms, tissues, organs, whole animals or whole plants, which method comprises:

(a) bringing the moiety to be preserved in contact with a thermal hysteresis protein in sufficient concentration to interact with the proteins, lipids, cell membranes, cells, microorganisms, tissues or organs;

(b) exposure to the nonphysiological conditions;

(c) optionally first removing the macromolecule;

(d) returning the proteins, lipids, cell membranes, cells (animal or plant), microorganisms, tissues or organs to a physiological temperature and composition, while optionally simultaneously removing the macromolecule; or optionally

(e) subsequently removing the thermal hysteresis protein after returning the biological material to the physiological temperature and composition.

In one embodiment, the temperatures are hypothermic, i.e., close to 0°C or lower and are used for preservation of proteins, lipids, cell membranes, cells (animal or plant), microorganisms, tissues, organs, animals or plants. For example, pig oocytes are preserved in such a way at about 4°C to 24 hr or more. Rat livers are preserved by this method at 4°C for 24 hr and preferably longer 48 hr, 96 hr, etc.

In another aspect the present invention related to a method for preservation of animal proteins, enzymes, lipids, cell membranes, cells, microorganisms, tissues or organs at

temperatures below 0°C to about 4K which method comprises:

(a) bringing the moiety to preserved in contact with the biologically compatible substance (e.g. AFP or AFGP in the presence of only an aqueous solution or with addition the other cryoprotective compounds such as glycerol, propylene glycol, etc.;

(b) cooling preferably to cryogenic temperatures (by such means as liquid nitrogen) and either vitrifying or freezing the system according to the various concentrations and cooling rates using higher concentrations of the additional compounds, such as propylene glycol or glycerol and high er cooling rates which lead to vitrification and to lower freezing temperatures (e.g. with 40% v/v propyleneglycol/water) and with a cooling rate of 1,750°C/min., vitrification is achieved);

(c) maintaining the proteins, lipids, cell membranes, cells, microorganisms, tissues or organs at these temperatures for periods of more than 24 hours, 7 days, 52 weeks or to about 10 years,

(d) warming, by such means as warm fluids or microwave heating, to physiological conditions, and

(e) removing the thermal hysteresis protein e.g. antifreeze glycoproteins and the other compounds, (e.g., by perfusion or flushing) and replacing them with physiological compatible solutions to regenerate the viable biological moiety.

For example, with 12.5% v/v, propylene glycol/water at a cooling rate of 1,200°C/min., ice crystals were formed. In all cases, viable mouse embryos and pig oocytes were obtained after exposure to -130°C for several hours;

Bovine oocytes, pig oocytes, pig embryos and mouse embryos survive this protocol in a aqueous composition of about 10 to 20 mg/ml antifreeze glycoproteins from Antarctic fish from the family Nothotheniidae.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A, 1B and 1C are transmission light micrographs of ice crystals (i), in aqueous solution is

frozen with a cooling rate of 4°C/min on a directional solidification stage, see U.S. Patent 4,531,373.

Figures 2A, 2B and 2C are scanning electron micrographs of liver tissue perfused with 40 mg/ml AFGPs (see definitions below) and frozen with a cooling rate of approximately 4000°C/min.

Figures 3A, 3B, 3C, 3D and 3E are scanning electron micrographs of frozen liver tissue.

Fig. 4 (4A to 4D) are photographs of the cryopreservation of immature pig oocytes.

Fig. 5 (5A to 5B) are photographs of the cryopreservation of pig embryos at the two-cell stage.

Fig. 6 (6A, 6B and 6C) are photographs concerned with cryopreservation of mouse embryos at the two-cell stage.

Fig. 7 (7A, 7B and 7C) are photographs showing with the hypothermic preservation of pig oocytes.

Figure 8 is a photographic representation of rat liver tissue (A7) at a magnification of about x 400. This tissue without AFGP treatment was cooled to -35°C at 21.5°C/min.

Figure 9 is a photographic representation of rat liver tissue at a magnification of about x 400. This liver tissue was flushed with a Krebs solution containing 20 mg/ml of AFGP fractions 1-8 (Table 1) at 37°C prior to cooling to -35° at 21.5°C/min.

Figure 10 is a graph of the bile production from whole rat liver treated with Krebs solution and Krebs solution AFGP as a function of time (see Example 7A).

Figure 11 is a graph of the LDH level from whole rat liver treated with Krebs solution and Krebs solution and AFGP as a function of time (see Example 7A).

Figure 12 in a graphic representation of the percentage of oocytes with normal membrane potential after hypothermic exposure for 4 hr at 4°C without and with various concentration AFGPs.

Figure 13 is a graphic representation of the percentage of oocytes with normal membrane potential after hypothermic exposure for 24 hr at 4°C without and with various

concentrations of AFGPs.

Figure 14 shows a graphic representation of the percentage of bovine oocytes with an intact oolemma for morphological microscopic examination (Morph), fluorescein diacetate staining (FDA) and trypan blue exclusion for a control, AFP winter flounder (WF), AFP ocean pout (OP) and AFP sea raven (SR).

Figure 15 shows a graphic representation of the percentage of bovine oocytes matured or fertilized in vitro for control, AFP winter flounder (WF), AFP ocean pout (OP), AFP sea raven (SR) and fresh.

Figure 16 is a graph of current versus time for superimposed raw currents showing the effect of AFP (0.5 mg/ml) on Ca^{+2} currents. Test pulses were given at 20 sec intervals. The currents shown are (a) before infusion (b) after 20 sec and (c) after 40 sec. Complete current suppression is achieved in 40 sec.

Figure 17 is a graph of current versus time for superimposed raw currents showing the effect of AFP (0.5 mg/ml) on K^+ currents. Test pulses were given at 20 sec intervals. To make the figure legible we show currents obtained, before infusion of AFP, curve (a), and then in subsequent 40 sec intervals. Complete current suppression is achieved in 200 sec.

Figure 18 is a graph of current versus time for superimposed raw currents showing the effect of AFP (10 mg/ml) on K^+ currents. Test pulses were given at 20 sec intervals. The currents shown are (a) before infusion of AFP, and (b) 20 sec after the infusion. Complete current suppression is achieved in 20 sec.

Figure 19 is a graphic representation of the effect of Cai concentration (nM) versus time (sec) in parietal cells.

Figure 20 is a graphic representation of the effect of the sequential treatment of parietal cells with 100 micro M carbachol in a control and with AFP versus time (sec).

Figure 21 is a graphic representation of the calcium internal store of Cai concentration (nanoM) for control and

with AFP versus time.

Figure 22 is a graphic representation of Cai concentration with short treatment of glad cells with AFP for a control and AFP treated cells versus time (sec).

DETAILED DESCRIPTION OF THE INVENTION AND PREFERRED EMBODIMENTDS

Definitions:

As used herein:

"Abnormal nonphysiological chemical conditions" refers to conditions different from the normal physiological conditions include, but are not limited to high or lowered temperature, freezing, excess or limited carbon dioxide, excess or limited oxygen, excess or limited inorganic salts, excess or limited organic compounds, different pH values radiation or combinations thereof.

"Antifreeze proteins" or "antifreeze polypeptides" ("AFP") or "antifreeze glycoproteins" or "antifreeze glycopeptides" (AFGP)" are macromolecules found in the body fluids of some animals (e.g. cold blooded) which have the commonly known property that they reduce non-colligatively the phase transition temperature of water by direct interaction with and inhibition of the growth of ice crystal nucleii that form at temperatures below the phase transition temperature.

Antifreeze compounds (from any source) are also known as "thermal hysteresis proteins" because while the phase transition temperature is apparently depressed during freezing by an amount much larger than the colligative effect of the molecule, it is not depressed during melting except to the extent caused by the colligative effect of the molecule. Prior to the present invention, this was the only known property of these antifreeze compounds. (Sources of antifreeze peptide (or protein) are described below).

"Cryogenic temperatures" refers in the area of cryobiology, below 0°C to as low as 4K or lower.

"Freezing" refers to condition (iii) is performed at -0.50°C and below to 4K. The rate of freezing is rapid,

about 1000°C/min or slow about 1°C min, preferably between about 4°C and 200°C/min especially about 4 to 50°C/min.

"Hyperthermic" refers to (condition iv) temperatures higher than the normal physiological temperature of a cell, tissue, organ, plant or animal, e.g. up to 20°C greater than physiological, preferably about 10°C greater, more preferably about 5°C greater.

"Hypothermic" refers to temperatures lower than the normal physiological temperature of a cell, tissue, organ or animal to about 0°C.

"Mammal" refers to any warm blooded mammal as generally defined, including, for example, pig, cow, rabbit, horse, human being, and preferably a human being.

"Non-physiological chemical or environmental conditions" refers to (condition v) excess or reduced oxygen (± 50%), excess or reduced carbon dioxide (± 50%) from physiological concentrations, having different ion concentrations from physiological (± 10% by weight, preferably ± 5% by weight, more preferably ±·1% by weight, pH values ± 3 pH units from physiological or a combination thereof.

"Optional" or "optionally" refers to the situation in which a component may or may not be present, or where a step may or may not be performed, within the scope of the invention.

"Prism planes" refer to another convention to describe the growing ice formation on an ice crystal. There exist secondary prism planes perpendicular to the a-axes and pyramidal planes that project off these planes. Crystallography terminology describes these planes in terms of the following pyramidal Miller-Bravais indices:

Primary prism plane	$(1\ 0\ \bar{1}\ 0)$
Secondary prism plane	$(1\ 1\ \bar{2}\ 0)$
Pyramidal pane from the primary prism plane	$(2\ 0\ \bar{2}\ \bar{1})$
Pyramidal plane from the secondary prism plane	$(1\ 1\ \bar{2}\ 1)$

Ice crystal growth under normal circumstances is along the a-axes. Ice crystal growth using the AFPs or AFGPs of the present invention is altered to be preferred in the direction of the c-axis.

For more information, see Peter V. Hobbs (1974) Ice Physics, Clarendon Press, Oxford, England, Appendix A etc., p. 725 ff.

"Rapid cooling" refers to a technique developed for long term preservation of cells and biological organs at cryogenic temperatures. The rapid cooling is used to produce very small, non-damaging ice crystals, see A. Trounson, (1986) Fertility and Sterility, Vol. 46, 1-12.

"Vitrification" refers to a technique for long term preservation of cells and biological organs at cryogenic temperatures. The technique involves introduction into the biological materials of different cryoprotective compounds such as glycerol, dimethylsulfoxide, propylene glycol, etc. which depress colligatively the phase transition temperature for water and increase its temperature. Next, the whole cell suspension or organ is rapidly cooled in the presence of the cryprotective compounds with the expectation that the water in the biological materials will remain polymorphous in a glass form and that no damaging ice crystals will occur or exist. (See Fahy, G.M. et al., Cryobiology, Vol. 21, 407-426, (1984), W.F., Rall and Rahy, G.M. Nature, Vol. 313, 573-575, 1985)). Rates of cooling for vitrification (condition ii of the invention) for small unorganized cells (e.g. ova, sperm, embryo) and for organs is rapid, about 100 to 2000°C/min, preferably about 200 to 1750°C/min, more preferably about 200 to 1000°C/min, especially about 200°C. The best cooling rate is determined by the objectives of the preservation.

Sources of Antifreeze Proteins

Antifreeze proteins (AP-which includes AFP and AFGP) were found first in the body fluids of marine teleost fish which are hypoosomotic, have a blood serum freezing point of -0.7°C, but inhabit the polar ice-laden waters (Scholander

et al. J. Cell Comp. Physiol., Vol. 49, 5-24, 1957). The first AP's were found by DeVries (Doctoral Thesis, Stanford, 1968) in Antarctic notothenиid fish. Two types of antifreeze proteins have been isolated from polar and temperate fish, glycopeptide and peptides. In studies of fishes with two exceptions, the antifreeze compounds are glycopeptides.

These antifreeze glycopeptides (glycoproteins) are present in eight distinct molecular weight classes ranging from - 2,500 to 34,000. They generally consist of a peptide backbone made-up of repeats of the tripeptide alanyl-alanyl-threonyl (the small glycopeptide may replace some alanines with proline beginning at position 7, with the disaccharide sugar beta-D-galactopyanosyl-(1→3)-2-acetamide-2-deoxy-alpha-D galactopyranose attached via a glycoside linkage to the hydroxyl side chain of each threonine (A. DeVries, Science, Vol. 172, 1152-1155, 1971).

These polypeptide or glycopolypeptides are available from a number of natural sources, e.g.: from body fluids of reptiles (e.g. turtles), invertebrates, insects, amphibians or fish. Preferably, the AFP's are obtained from the serum or body fluids of Arctic, Antarctic, North Temperate or South Temperate fish. More preferably, the serum and fluids of Arctic or Antarctic fish are used, e.g. See Table 1 below.

TABLE 1

MOLECULAR WEIGHTS OF ANTIFREEZE GLYCOPEPTIDES

A. Antifreeze glycopeptide isolated from the Antarctic notothenidae are fish; Pagothenia (Trematomus) borchgrevinki.

Antifreeze Glycopeptide Fraction No.	Molecular Weight (Approx.)
1	33,700
2	28,800
3	21,500
4	17,000
5	10,500
6	7,900
7	3,500
8	2,600

Glycopeptide from Dissostichus mawsoni of structure

Ala-Ala-Thr-Ala-Ala-Thr-Ala-Ala-
NAGA NAGA
GAL GAL

Thr-Ala-Ala-Thr-Ala-Ala-Thr-Ala-Ala-
NAGA NAGA NAGA
GAL GAL GAL

The molecular weights of the fractions are essentially identical to those of Table 1 above.

AFGPs isolated from the blood of Antarctic notothenid fish exist in at least 8 sizes depending on the number of repeats of the basic glycotripeptide unit, see Table 1. The molecular weight ranges between 2,600 and 33,700 (DeVries et al. (1970)) The AFGP's make up 3-4% of the blood of the fish and along with the sodium chloride, they lower the fish's freezing points below that of seawater. The AFTPs inhibit the growth of ice crystals by adsorption to the ice crystal (Raymond et al. (1977), DeVries (1984)). Adsorption occurs on specific faces of the ice crystal (primary prism face (1010)) resulting in inhibition of ice growth on these faces (DeVries, 1984), Consequently, in solutions of AFGPs,

ice crystals grow predominantly on the basal plane (parallel to the c-axis), to which the AFGPs do not absorb, and take the form of very small, needle-like ice crystals (Raymond et al., (1977); DeVries, 1988)). Peptide antifreezes can be found in several North Temperate, Arctic or Antarctic fish. The peptides vary in size and composition.

These polypeptide are essentially different lengths of the repeating tripeptide -alanine-alanine-threonine- where substantially each threonine is joined by a glycosidic linkage to the disaccharide B-D-galacto-pyransoyl-(1→3)- 2-acetamido-2-deoxy-alpha-galactopyranose.

The small glycopeptide may also have a small amount of proline located at positions 7, 10 and 13, but are otherwise structurally the same as the large glycopeptide.

Generally the higher the molecular weight, the more effective is the antifreeze glycopeptide in promoting ice crystal growth along the c-axis, for example, fractions 1-5 above individually or as a mixture as obtained by purification. Fractions 6, 7 and 8, having a lower molecular weight, individually or as a group are apparently less effective in preservation.

The antifreeze glycopeptides are essentially similar in all the Antarctic Nototheniidae fish including (Pagothenia borchgrevinki, Trematomis Nicolia, Dissostichus Mawsoni (J.T. Eastman, and A.L.DeVries, A.L. Scientific American, Vol. 254, 106-114 (1986)). The same eight glycopeptides have also been isolated from northern hemisphere gadid, the rock cod (Gadus ogac) and in some other northern cods belonging to the family Gadidae (DeVries, A.L. Comp. Biochem. Physiol., Vol. 90B, No. 3, pp. 611-621 (1988). All the AFGP's isolated so far are similar in structure with relatively small changes such as the position occupied by proline in antifreeze glycopeptide 8 in northern species, or difference in size in northern cod, but essentially the same composition (A.L. DeVries, (1984) Phil. Trans. R. Socl. Lond., Vol. B 304, 575-588).

The other kind of antifreeze proteins found in fish are

polypeptides. While the antifreeze glycopeptide are in general polymers of a glycotripeptide unit Ala-Ala-Thr with disaccharide linked to the Thr side chain, the peptides are quite diverse structurally and vary in size and composition.

The antifreeze protein from the winter flounder, Pseudopleuronectes americanus, although having a specific activity similar to that of the larger molecular weight glycoproteins, lacks sugars entirely and instead has high percentages of hydrophilic amino acids (especially threonine and Asp) while still retaining a large amount (about 60 mol %) of alanine. The flounder protein primary structure has clusters of hydrophilic amino acids separated by sequences of alanine, (Duman and DeVries (1976) Comp. Biochem. Physiol, Vol. 533, 375-380).

Peptides from Winter Flounder

Asp-Thr-Ala-Ser-Asp-
Ala-Ala-Ala-Ala-Ala-Ala-Leu-Thr-Ala-Ala-Asp-
Ala-Ala-Ala-Ala-Ala-Ala-Leu-Thr-Ala-Ala-Asp-
Ala-Ala-Ala-Ala-Ala-Ala-Ala-Thr-Ala-Ala.

Origins of the Biologically Compatible Substance.

Peptides from the Antarctic eel pout Rhigophila dearborni

Peptide No.	Molecular Weight
1,2,3 (three components)	6,900

Asn-Lys-Ser-Val-Val-Ala-Asn-Gln-Leu-Ile-Pro-Ile-Asn-Thr-Ala-Leu-Thr-Leu-Ile-Met-Lys-Ala-Glu-Val-Val-Thr-Pro-Met-Gly-Ile-Pro-Ala-Glu-Asp-Ile-Pro-Arg-Ile-Ile-Gly-Met-Gln-Val-Asn-Arg-Ala-Val-Pro-Leu-Gly-Thr-Tyr-Leu-Met-Pro-Asp-Met-Val-Lys-Asn-Tyr-Glu-.

Other fish that produce antifreeze peptides are listed in A.L. DeVries, Phil. Froms. R. Soc. London, Vol. 304, 575-588 (1984) such as the Alaskan plaice, Atlantic sculpins, Grubby Sculpin (Yang, D.S.C. Nature, Vol. 333, 232-237, 1988) and the Antarctic Eelpout (Rhigophila dearborni). Recent reviews of the antifreeze proteins in fishes can be also found in (Feeney and Burchan (1986), Ann.Rev. Biophys. Biophys. Chem., Vol 15, 53-78,) and (Davies et al. (1988)

Canadian J. Zool, Vol. 66, 2611-2617).

V.S. Ananthanarayanan, Life Chemistry Reports, Vol. 7, pp. 1-32 (1989), also describes sources of antifreeze protein, particularly Type I, II and III. See Table 1A below.

TABLE 1A

TYPES OF FISH ANTIFREEZE PROTEIN

Type, composition and size	*Fish species*	*Trivial name*
Antifreeze glycoproteins (AFGPs): contain alanine, threonine and Gal-GalNAc disaccharide; M_r: 2,600–33,700	Antarctic notothenioids:	
	Pagothenia borchgrevinki	
	Trematomus borchgrevinki	Antarctic cod
	Dissostichus mawsoni	
	Northern ocean gadoids:	
	Gadus agac	Greenland cod
	Gadus morhua	Atlantic cod
	Microgadus tomcod	Atlantic tomcod
	Boreogadus saida	Arctic polar cod
	Eligenus gracilis	Saffron cod
Antifreeze Polypeptides (AFPs): Type I, alanine-rich; M_r: 3,300–6,000	Righteye flounders:	Winter flounder
	Pseudopleuronecus americanus	
	Limanda ferruginea	Yellowtail flounder
	Cottids:	
	Myoxycephalus scorpius	Shorthorn sculpin
	Myoxocephalus aenaeus	Grubby sculpin
	Myoxocephalus scorpiodes	Arctin sculpin
Type II, cysteine-rich; M_r: 14,000–16,000	Cottid:	
	Hemitripterus americanus	Sea raven
Type III, no cysteines and are not rich in alanine, M_r: 5,000–6,700	Eel pouts:	
	Macrozoarces americanus	Ocean pout
	Rhigophila dearborni	Antarctic eel pout
	Lycodes polaris	Arctic eel pout

These AFPs, AFGP's (or fractions and mixtures of fractions thereof) and others are available upon request from Dr. Arthur DeVries, Department of Physiology, Burrill Hall, 407 S. Goodwin, University of Illinois, Urbana, IL 61801.

These antifreeze proteins or peptides of the present invention may also be produced by synthetic means. These means include the use of a peptide synthesizer available commercially in the art as Model 430A, Applied Biosystems, Inc., Foster City, California. The operation manuals for this peptide synthesizer are useful. The synthesis procedures of J.J. Nestor, et al., U.S. Patent 4,318,905, and R.B. Merrifield, U.S. Patent 3,531,258 are specifically incorporated herein by reference and are adapted for the preparation of the Ala-Ala-Thr and Ala-Ala-Ala compounds described above. Once the peptide is prepared, the threonine residues are optionally bonded to the disaccharide by conventional methods.

The antifreeze protein of the present invention are independently selected from the protein themselves, or glycoprotein, or the protein or glycoprotein covalently bonded to a carrier such as biologically compatible antibody, gelatin, biocompatible polymer, peptide, sugar, or carbohydrate. Mixtures of these antifreeze materials are contemplated and are part of the present invention. Covalent bonding of a protein to a carrier by methods known to those of ordinary skill in the art are, for example, found in K. Rubenstein, et al., U.S. Patent 3,817,837, or M. Goodman et al., U.S. Patent 4,837,305, which are specifically incorporated herein by reference in their entirety.

Recombinant DNA Production of Antifreeze Polypeptide

It is also contemplated within this present invention to produce compositions wherein the peptides (amino acid sequences) are produced by recombinant DNA technology. The DNA sequences encoding these genes have been elucidated. See, for example, A.L. DeVries et al. (1971), J. Biol.

Chem., Vol. 246, p. 305; Y. Lin, et al. (1972), Biochem. Biophys. Res. Commun., Vol. 46, p. 87; D.S.C. Yang et al. (1988), Nature, Vol. 333, p. 232; Y. Lin (1981), Proc. Natl. Acad. Sci. U.S.A, Vol. 78, p. 2825; P.L. Davies et al. (1982), J. Biol. Chem., Vol. 79, p. 335; B. Gourlie et al. (1984), J. Biol. Chem., Vol. 259, p. 14960; P.L. Davies et al., J. Biol. Chem., p. 9241; G.K. Scott et al. (1986), Can. J. Fish. Aquat. Sci., Vol. 43, p. 1028; G.K. Scott et al. (1988), J. Mol. Evol., Vol. 27, p. 29. Microinjection of the AFP gene into other species has been successful. See for example, Z. Zhu et al. (1985), Angew. Ichtoyol, Vol. 1, p. 31; Kexue Tongbao (1986), Vol. 31, p. 988; D. Chourrout et al. (1986), Aquaculture, Vol. 51, p. 143; R.A. Dunhan et al. (1987), Trans. Am. Fish. Soc., Vol. 116, p. 87; G.L. Fletcher et al. (1988), Can. J. Fish Aquat. Sci., Vol. 45, p. 352; N.D. Maclean et al. (1987), Bio Technology, Vol. 5, p. 257; G.W. Stuart et al. (1988), Development, Vol. 103, p. 403; T. McEvoy et al. (1988), Aquaculture, Vol. 68, p. 27; K. Ozato et al. (1986), Cell Differ., Vol. 19, p. 237; T.T. Chen et al. (1989), UCLA Symposium on Transgenic Animals; T.T. Chen et al. (1989), Aquaculture; P. Zhang et al. (1989), Mol. Reprod. Dev.; D.A. Powers et al. (1989), NIH Symposium on Transgenic Animals. The general formation of the DNA sequences to produce protein is found in the following U.S. Patents 4,237,224; 4,708,948; 4,376,071; 4,350,687; 4,444,760 and 4,722,998. The procedures are adapted to produce AFPs. All of these references are specifically incorporated herein by reference.

Recently antifreeze proteins (thermal hystersis protein) which is useful in the present invention were also found in many invertebrates. A list of these invertebrates is given in Table 2 and 3 with the references, found in the tables following immediately after the tables.

TABLE 2

Thermal Hysteresis Protein Producing Invertebrates

A. Insects (minus beetles).

Order	Species	Reference
Collembola	7 spp.	Zettel, 1984
Plecoptera	Arcynopteryx compacta	Gehrken and Somme, 1987
Orthoptera	Parcoblata pennsylvanica	Duman, 1979
Hemiptera	Oncopeltus Fasciatus	Paterson et al. 1981
Mecoptera	Boreus westwoodi	Husby and Zacharissen, 1980
Lepidoptera	Choristoneura fumiferana	Hew et al., 1983

B. Coleoptera (Beetles)

Family	Species	Reference
Tenebrionidae	Tenebrio molitor	Ramsay, 1964 Patterson and Duman, 1978
	Meracantha contracta	Duman, 1977a
	Uloma impressa	Duman, 1979
	Platydema sp	Duman, 1979
Elateridae	Ampedus lineatus	Duman, 1979
	Ampedus sp	Duman, 1979
	Lepidotus discoideus	Duman, 1979
	Melanotus sp	Duman, 1979
Cucjidae	Cucujus clavipes	Duman, 1979
Pyrochridae	Dendroides canadensis	Duman, 1979, 1980

Lampyridae	Photinus sp	Duman et al., 1982
Coccinellidae	Coccinella novemnotata	Duman et al., 1982
Scolytidae	Ips acuminatus	Gehrken, 1984
Cerambycidae	Rhagium inquisitor	Bremdal and Zachariassen, 1988

C. Non-Insect Arthropods

Animal	Species	Reference
Spiders	Philodromus sp	Duman, 1979
	Clubiona sp	Duman, 1979
	Bolyphantes index	Husby and Zachariassen, 1980
Centipede	Lithobius forficatus	Duman et al., 1982 Tursman and Duman, unpublished
Mite Duman,	Alaskozetes antarcticus	Block and 1989

D. Other Invertebrates.

Mussel	Mytilus edulis	Theede et al., 1976

TABLE 3

Amino Acid Compositions of Representative Insect THP's

(Values are in Mol %)

Amino Acid	-1[a]	T-4[b]	T-3[c]	Budworm[d]	Carudensis[d]
Asx	11.3	7.3	5.3	9.5	14.3
Thr	11.0	6.6	2.3	6.0	17.2
Ser	14.8	7.4	11.1	13.0	10.3
Glx	15.3	8.9	12.4	11.0	5.2
Pro	5.9	5.9	0.0	5.0	2.6
Gly	7.6	8.3	11.4	15.0	6.5
Ala	9.6	14.3	5.0	8.0	8.4
1/2Cys	0.0	0.0	28.0	6.0	15.9
Val	7.2	11.5	2.3	3.0	1.7
Met	0.0	4.8	0.0	0.0	0.2
Ile	3.3	7.1	1.0	1.2	1.5
Leu	3.9	0.0	2.2	6.5	1.9
Lys	4.8	6.8	15.4	3.1	3.4
Arg	1.1	2.6	0.0	8.0	4.8
Tyr	1.2	2.3	0.0	1.0	3.9
Phe	1.5	3.9	0.0	2.2	0.0
His	1.5	1.9	3.1	0.0	1.9
%Hydrophilics[f]	58.3	40.0	46.5	50.6	55.2

a Patterson and Duman, 1979

Table 3 continued

b Tomchaney et al., 1982

c Patterson and Duman, 1982

d Hew et al., 1981

e Wu and Duman, unpublished

f The percentage of amino acid residues with hydrophilic side chains (Asx, Glx, Lys, Arg, Ser, Thr), according to groupings of Manavalan and Ponnuswamy (1978).

References pertaining to Tables 2 and 3:

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D.E. Rancourt, et. al. (987) Mol. Cell Biol., Vol. 7, pp. 2188-2195.

R. Schneppenheim and H. Theede (1980) Comp. Biochem. Physiol. B. Vol. 67, pp. 561-568.

D.G. Slaughter, et. al. (1981) J. Biol. Chem., Vol. 256, pp. 2022-2026.

L. Somme (1978) Norw. J. Ent., Vol. 25, pp. 187-188.

A.P. Tomchaney, et. al. (1982) Biochem., Vol. 21, pp. 716-721.

J. Zettel (1984) Rev. Ecol. Biol. Sol., Vol. 21, pp. 189.

GENERAL

In the present invention using aqueous antifreeze protein compositions, the process of ice freezing in tissue is changed, the viscosity of liquid contacting the membrane is increased, and the structural damage to the tissue reduced or eliminated through modification of the pattern of ice crystal growth. This advance is accomplished by modifying the pattern of ice crystal growth in tissue using novel compositions, e.g., peptides or glycopeptides from Arctic or Antarctic fish or from other sources. The effect of the antifreeze proteins on the freezing pattern in aqueous solutions is documented extensively as cited above. The different antifreeze proteins from different sources adsorb to different crystal faces, however, all antifreeze protein inhibit ice crystal growth parallel to the a-axes, thermodynamically preferred direction of growth. Freezing in the presence of any kind of antifreeze protein invariably

leads to ice crystals forming in the direction of the c-axis. The ice crystals grow in the form of spicules (spikelike structures). These small micron and submicron scale spicular structures are stable and entrap between them the solutes that are rejected during formation of ice.

SOLUTION PREPARATION

The aqueous solution THP (e.g. AFP or APGP) compositions are prepared by any number of methods. Water (usually sterile) is contacted with THP and mixed to produce a solution of between about 0.01 and 100 mg/ml solute in water. Usually the antifreeze protein saturates in water in concentrations greater than about 100 mg/ml. Preferably, a solution of between about 1 and 60 mg AP/ml is produced, especically between about 10 to 40 mg/ml, more preferably about 1 to 20 mg/ml. The aqueous phase may also contain salts, sugars, ions nutrients (e.g. Krebs solution) and mixtures thereof in concentrations known in the art to be useful for preserving biological agents. The aqueous phase may also contain other materials, e.g. glycerol, etc., which are useful in the preservation of tissue, cell membrane, etc.

A number of electrolyte solutions useful as biologically compatible aqueous solutions are known and described in the literature for short term prreservaiton of organs an tissue. R.L. Veech is U.S. Patent 4,663,289 (which is incorporated by reference) discloses a number of processes and compositions for in vitro use in tissue culture media, perfusion media and incubation media. See for Example Tables 4 and 5 below. Balanced salt solutions are disclosed which include but are not limited to selected from sterile normal plasma, normal saline containing 0.9% by weight sodium chloride, normal saline containing 0.95% by weight sodium chloride, Ringers solution, mammalian Ringer UK and Canada solution, lactated Ringer's solution, acetated Ringer's solution, Locke's solution, Tyrode's solution, Krebs solution, Krebs-Henseleit solution, Krebs Ringer phosphate solution, Krebs serum substitute solution, Krebs

Improved Ringer II solution (calcium ion free), or Krebs Improved Ringer III (low bicarbonate, low phosphorus), Schumarach liver solution, Krebs kidney perfusion solution, Bahlman kidney perfusion solution, University of Wisconsin preservation solution, Collins solution, EuroCollins solution, Ross-Marshall solutions purified plasma from the animal from which the organ or tissue is obtained, Fulgaff perfusion solution, and Wikman-Coffelt solution and combinations thereof.

The thermal hysteresis protein is present in these solutions in the concentrations shown immediately above.

TABLE 4

TABLE I

Prior Art Simulated Plasma Electrolyte Solutions for Contacting Living Cells in Vitro.

Units mmoles L fluid	Normal Plasma N E J.M. 283, 1285 1970	(1) Normal Saline 0.9% NaCl U.S.	(2) Normal Saline 0.95% NaCl U.K.	(3) Ringer's Injection U.S.	(4) Mammalian Ringer's U.K. & Canada	(5) Lactated Ringer's U.S.	(6) Lactated Ringer's (Hartmann)	(7) Acetated Ringer's	(8) Locke's	(9) Tyrode's	(10) Krebs Henseleit	(11) Krebs Ringer Phosphate	(12) Krebs Serum Substitute	(13) Krebs Improved Ringer II Ca^{2+} free	(14) Krebs Improved Ringer III Low HCO_3 Low Pi
Na	136-145	154	162.5	147	156.4	130	129.8	130	157.57	150.1	143	150.76	140.31	147.41	140.86
K	3.5-5.0			4	5.4	4	5.4	4	3.57	5.9	5.9	5.92	5.92	5.92	5.92
Ca	2.1-2.6			2.5	1.15	1.5	0.9	1.5	2.16	1.8	2.5	2.54	2.54		2.54
free [Ca^{2+}]	[1.06]														
Mg	0.75-1.25														
free [Mg^{2+}]	[0.53]						1.0			0.45	1.2	1.18	1.18	1.18	1.18
Σ mEq Cations	142.7-153.2	154	162.5	156	164.1	137	139	137	165.46	160.5	156.3	164.12	153.7	155.69	154.22
Cl	100-106	154	162.5	; 156	161.7	109	111.8	109	163.92	147.48	127.8	131.51	104.62	103.06	122.36
HCO_3	26-28				2.4				3.57	11.9	25		24.9	3.56	3.56
Σ Pi	1-1.45									1.22	1.18	17.38	1.18	15.03	3.49
SO_4	0.32-0.94										1.18	1.18	1.18	1.18	1.18
L-lactate	0.6-1.8					28(d,l)	27.2(d,l)								
pyruvate													4.92	4.92	4.92
Lact/pyr															
D-β-OHbutyrate															
acetoacetate															
β-HB/acac															
acetate															
Other								28				fumarate²⁻	5.3°	5.39	5.39
												glutamate⁻	4.92	4.92	4.92
Σ mEq anions	128.7-139.4	154	162.5	156	164.1	137	139	137	167.49	161.6	157.3	163.97	152.49	156.64	155.17
Na/Cl	1.28-1.45	1.00	1.00	0.94	0.97	1.23	1.16	1.19	0.96	1.02	1.12	1.15	1.34	1.43	1.15
Glucose or others	3.9-5.6								5.6-13.9	5.6			11.5	11.5	11.5
CO_2	0.99-1.39										1.24		1.24		
pH	7.35-7.45	≈5.5-6.5	≈5.5-6.5	≈5.5-6.5	≈7.0					7.1	7.4	7.4	7.4	≈7.6	≈7.6
Σ mOsm	285-295	308	325	309	324	272.5	276	272.5	336	318.8	308	311.7	309.8	304.1	307.8
Use															

Footnotes for Table 4:

(1) Usual "physiological saline" in the U.S. is a 0.9% or 154 mM. [Gilman AG, Goodman LS, Gilman A. The Pharmacological Basis of Therapeutics ((1980) pp 848-884, MacMillan, London
(2) "Physiological Saline" in the U.K. is 0.95% NaCl [Diem K. ed. Documenta Geigy (1962) pp 333-334, Geigy, Manchester
(3) All "Ringer's solutions" are derived from Ringer S. Physiol 4, 29, & 222, 1893 and 7, 1886. This commercial U.S. version is from Facts and Comparisons, Oct 1981, p. 50, Lippincott, St. Louis
(4) From Best and Taylor, Physiological Basis of Medical Practice, 6th edition, Baltimore, 1950
(5) From Facts and Comparisons p. 50, Oct '81, Lippincott, St. Louis
(6) Hartmann AF. J Am Med Assoc 103: 1349-1354, 1934.
(7) Fox CL, et al. J Am Med Assoc 148: 825-833, 1952.
(8) Locke FS. Zbl Physiol 8,166, 1894; 14, 670, 1900; 15, 490, 1901.
(9) Tyrode MJ. Arch Int Pharmacodyn 20, 205, 1910.
(10) Krebs HA, Henseleit KA. Hoppe-Seyle's Z Physiol Chem 210, 33-66, 1932
(11) Krebs HA. Hoppe-Seyle's Z Physiol Chem 217, 193, 1933
(12) (14) Krebs HA. Biochem Biophys Acta 4, 249-269, 1950.

TABLE 5

	"Prior Art Perfusion Fluids"						
Units mmoles L fluid	Normal Plasma N...J.M. 283, 1285 1970	(15) Krebs Liver Perfusion with Bovine serum Albumen and Red Cells	(16) Schimassek Liver Perfusion	(17) Krebs Kidney Perfusion	(18) Hepatocyte Incubation	(19) Bahlman Kidney Perfusion	(20) Fulgraff Kidney Perfusion
Na	136–145	153	151.54	148	153	147	143
K	3.5–5.0	5.9	5.9	5.9	5.9	4.9	4.74
Ca	2.1–2.6	2.5	1.8	2.5	2.5	2.56	1.25
free [Ca^{2+}]	[1.06]						
Mg	0.75–1.25	1.2	0.49	1.2	1.2	1.2	0.59
free [Mg^{2+}]	[0.53]						
Σ mEq Cations	142.7–153.2	166.3	162.02	161.3	166.3	159 4	151.15
Cl	100–106	127.8	147.48	127.8	127.8	127	113.04
HCO_3	26–28	25	11.9	25	25	24.5	25
Σ Pi		1–1.45	1.18	1.18	1.18	1.18	1.18
SO_4	0.32–0.94	1.18	—	1.2	1.2	1.18	1.18
L-lactate	0.6–1.8	(10Na–1Lac)	1.33	5Na–1Lac	9.09	2.75(d.l)	3.5(d.l)
pyruvate			0.09		0.91	0.25	0.25
Lact/pyr			14 8		10	10	7 or 14
D-β-OHbutyrate							
acetoacetate							
β-HB/acac							
acetate							5.0
Other							
Σ mEq anions	128.7–139 4	167.0	162.81	162.3	167.0	159 1	151.31
Na/Cl	1.28–1.45	1.12 (1.20)	1.03	1.16	1.20	1.20	1.26
Glucose	3.9–5.6		5.45			6.2	—
+ others						6.7 urea	6.7 urea
CO_2	0.99–1.39	1.25	1.24	1.24	1.24	1.24	1.24
pH	7.35–7.45	7.4	7.1	7.4	7.4	7.4	7.4
Σ mOsm	285–295	328	321	318	328	327	307.9
Albumin (g %)	3.5–5	3.9	2.5	5	2.5	5.5	0.05

[a] Artificial perfusion fluid generally add 1.3 to 5 g % albumin, dialyzed against a medium listed as Table I, that is Krebs-Henseleit [10], Krebs-Ringer Phosphate [11], Tyrode's [9], Locke's [8], or Krebs-Henseleit with a lowered Ca^{2+} to the 1 mM range, particularly in heart perfusion. They may or may not contain red cells. Krebs-Henseleit is known to contain about twice the amount of ionized Ca^{2+} as serum.

[15] Hems R, Ross BD, Berry MN, Krebs HA. Biochem J 101, 284, 1966. Krebs-Henseleit [10] with 3.9 g % bovine albumin

[16] Schimassek H. Biochem Z 338, 460, 1963. Essentially Tyrode's [9] with added lactate and pyruvate

[17] Nishiitsutsuji-Uwo JM, Ross BD, Krebs HA. Biochem J 101, 852–862, 1967. Krebs-Henseleit [10] with 5 g % albumin, dry

[18] Crow KE, Cornell NW, Veech RL. Biochem J 172, 29–36, 1978. Krebs-Henseleit [10] with 2.5 g % dialyzed albumin plus L-lactate plus pyruvate

[19] Bahlman J et al. Am J Physiol 212, 77 1967. Krebs-Henseleit [10] with lactate and pyruvate and 5.5 g % bovine albumin

[20] Fulgraff et al. Arch int Pharmacodyn 372, 49, 1972. Krebs-Henseleit [10] with 1 Mg and Ca plus lactate and pyruvate, plus 5 mM acetate, plus 0.05 g % albumin plus 2 g % hemoglobin

The H.A. Krebs K-Henseleit solution is known as an aqueous solution for viable organ tissue preservation. See Zeit. Physio Chemie, Vol. 210, pp. 33-66 (1932).

The composition in higher mammals is described as follows: Isotonic serum fluid

0.9%	NaCl	(0.15 mol (m))
1.15%	KCl	(0.154m)
	$CaCl_2$	(0.11 m)
2.11%	KH_2PO_4	(0.154 m)
3.82%	$MgSO_4$ $7H_2O$	(0.154 m)
1.3%	$NaHCO_3$	(0.154 m)

with 5 Vol % carbonic acid (H_2CO_3) to obtain a pH of about 7.4.

The Krebs solution of this reference has the following composition:

ION	AMOUNT	(mg-Percent)
Na+	327	mg-percent
K+	23	"
Ca++	10	"
Mg++	2,9	"
Cl-	454	"
PO_4=	11	"
SO_4"	11.4	"
HCO_3-	54	Vol.-percent
CO_2 (38°)	2.5	Vol.-percent
pH		7.4

with 5 Vol % carbonic acid (H_2CO_3) to obtain pH of about 7.4.

The Krebs solution of the present invention has the above composition to within ±0.1 mg-percent, and, preferably between ±0.05 mg-percent. especially ±0.01 mg-percent. Minor deviations are usually not of consequence for effectiveness of this solution.

Of the possible preservation solutions available, the Krebs solution was initially selected because of its weak preservation effect. Therefore, any preservation of cells, membranes and/or functional viability of membranes organs, etc. would be attributed to presence of the THP (e.g., AFP or AFGP).

J. Wikmann-Coffelt teaches additional preservation compositions for mammalian organs and tissue in U.S. Patent

No. 5,066,778, and specifically for mammalian heart in U.S. Patent No. 5,075,210.

For use in organ transplantation and the like, sterile conditions and solutions must be used. The solutions may be prepared using sterile materials and sterile conditions. Also the solutions may be sterilized by methods known in the art, e.g. brief exposure to cobalt-60 radiation.

TISSUE PRESERVATION

To illustrate the effect of AFPs on the ice crystal structure, experimental results of the present invention are presented from earlier research in which the freezing pattern in a physiological saline solution is compared to the freezing pattern in a physiological solution with the addition of between about 1-100 mg/ml preferably about 40 mg/ml of antifreeze glycopeptides from Antarctic notothenii ndae fish (Table 1). In this comparison, samples are frozen under controlled thermal conditions on a directional solidification stage. The directional solidification stage, described in greater detail in U.S. Patent 4,531,373 is an apparatus capable of freezing solutions or tissue samples with uniform cooling rates, between predetermined temperatures. The apparatus is used in conjunction with a light microscope to produce results shown in Figures 1A, 1B and 1C which demonstrate the spicular growth in the presence of antifreeze glycoproteins.

One embodiment of the present invention is to perfuse solutions containing antifreeze proteins through the vasculature of an organ. Upon freezing, the ice crystals that form will be small, spicular and will entrap the solutes present. Consequently the cells will not be exposed to high saline concentrations, and the damaging expansion of the blood vessels will be eliminated. This effect will be demonstrated with detailed experimental results using the directional stage and scanning electron microscope in Figures 2A, 2B, 2C and 3A and 3B, 3C, 3D and 3E.

VITRIFICATION

It was formerly observed that cells, tissue or organs

may not survive freezing with rapid cooling or "apparent vitrification". The expression "apparent vitrification" is used here to describe the observation that, at times, a solution is considered to be vitrified if it remains transparent after rapid cooling to cryogenic temperatures. However, the property of transparency is only an indication that the ice crystals are either too small or too few to reflect light and therefore, the vitrification is only apparent. In one aspect of the invention, it was expected that cells (or organs, tissue, animals) preserved by techniques in which the solution containing the cells (or organs, tissue, animals) is frozen by rapid cooling or apparent vitrification may be damaged by the preferential formation of very small ice crystals on the cell membrane, which may serve as a nucleation site. The antifreeze glycopeptides and peptides inhibit the growth of ice crystals and significantly reduce the size of the crystals formed by generating spicular ice structures. Therefore, these biologically compatible substances probably enhance the effectiveness of cryopreservation by preventing the formation of ice crystals on the cell membrane or by reducing the size of these ice crystals.

Pig Oocyte and Embryo Preservation

The effectiveness of the antifreeze proteins (in vitrification) was evaluated on the cryopreservation of immature pig oocytes, two-cell stage pig embryos and mouse embryos, at the two-cell stage frozen by rapid cooling and "apparent vitrification." Pig oocytes and pig embryos at the two-cell stage were chosen because they present a very challenging model for which no successful cryopreservation has been heretofore achieved. In fact, pig oocytes and early-stage pig embryos usually cannot survive exposure to temperatures as high as 10°C for even brief time periods.

The probability of ice crystal nucleation during cooling is an inverse function of viscosity and temperature and a direct function of volume (D. Turnbull, 1969). In cryopreservation by rapid cooling, attempts are made to

reduce the probability for nucleation by increasing the solution viscosity and by reducing the phase transition temperature through an increase in the concentration of various cryoprotectants. However, higher concentrations of cryoprotectants have a damaging effect on biological materials and, therefore, a proper balance must be found between a concentration that is sufficiently high to suppress nucleation and sufficiently low to avoid damaging the fragile cells.

These experiments were performed by exposing droplets of different size and composition to a variety of cooling rates on a special experimental system developed by B. Rubinsky, U.S. Patent 4,531,373. Rapid cooling, as well as rapid warming of samples, was performed using a Leitz Diaplan microscope to which a special directional stage was attached (A. Arav et al., 1990; B. Rubinsky, 1985; B. Rubinsky et al., 1985). The stage allows accurate control of cooling and warming rates between predetermined temperatures particularly as it is applied to vitrification and freezing by rapid cooling. A video camera was used in conjunction with the microscope to evaluate the morphology of the cells and the physical state of the solution.

An "apparent vitrification solution", (AVS) was useful which contains 17.5% propylene glycol, (Fluka Chemicals, Switzerland), 2.5% glycerol (BDH Analar, England), 20% FCS (Fetal Calf Serum) (Gibco, Scotland) and 0.05 M sucrose in PBS (Dulbecco's phosphate buffered saline supplemented with 0.4 m/v BSA (Bovine Serum Albumin), 0.34 mM pyruvate, 5.5 mM glucose and 70 μg/ml kanamycin).

This solution is physiologically compatible with mouse and pig embryos and with pig oocytes. When 0.1 μl droplets of the AVS solution were cooled at the rate of 1,700° C/min (the highest rate possible with the directional solidification stage) to a temperature of -130° C (a temperature lower than the glass formation temperature for this solution) no ice crystals were observed through the microscope at 340x magnification. To illustrate the effect

of volume and solute concentration, ice crystals were observed with all droplets of the AVS solution larger than 0.5 μl, and with all 0.1 μl droplets containing 12.5% propylene glycol and 2.5% glycerol when cooled at 1,700° C/min. No apparent devitrification, (that is, the formation of ice crystals was observed with droplets of the AVS solution larger than 0.5 μl, and with all 0.1 μl droplets containing 12.5% propylene glycol and 2.5% glycerol when cooled at 1,700° C/min.) No apparent devitrification (i.e., ice crystal formation) was observed when the samples were held at -130° C. However, devitrification was observed in some samples during warming to room temperatures even when the rate was as high as 1,700° C/min. The addition of AFGP's or AFP's was the AVS solution did not preclude the seldom and random occurrence of devitrification after "apparent vitrification". The AVS was the basic solution used in the experiment are reported to evaluate the effects of freezing with rapid cooling for used droplets larger than 0.5ml and for "apparent vitrification" droplets of 0.1ml. In the vitrification studies, only results from solutions that did not undergo devitrification were evaluated.

Evaluation of Cryoprotective Properties of AFPG & AFP in Oocytes and Embryos

To evaluate the cryoprotective properties of the AFGP's and the AFP's, immature pig oocytes, two-cell stage pig embryos and two-cell stage mouse embryos were introduced into either 0.1 ml droplets for vitrification, or droplets larger than 0.5 ml for freezing with rapid cooling of AVS with, and without, AFGP or AFP. These droplets were cooled on the directional stage under microscope observation at the rate of 1,700° C/min to -130° C. After 15 minutes at these temperatures, the samples were warmed at the rate of 1,700° C/min to room temperature. The survival of the embryos and oocytes was evaluated by in vitro culture followed by morphological and development analysis. Control experiments were performed by exposing embryos and oocytes to the different solutions in protocols identical to the rapid

cooling experiments, but without cooling and warming, and evaluating their viability. The glycopeptides used in this work were obtained from Antarctic fish belonging to the family Nototheniidae (Dissostichus Mawsoni) (Table 1). A physiological composition was used which consists of one part of fraction 1 to 5 (high molecular weights) and two parts of fraction 7 and 8 (lower molecular weights) as obtained form A. DeVries, University of Illinois. Fractions 1-5 are obtained as a mixture, and fraction 7-8 are obtained as a mixture. Experiments were performed with solution concentrations of 40 mg/ml glycopeptides. PBS is a standard buffered solution. This particular value was chosen because studies have shown that the depression of the freezing point of aqueous solutions of antifreeze glycoproteins is concentration-dependent and at these concentrations, it reaches saturation. A.L. DeVries, (1988).

After the cryoprotective properties of the AFGP's were established, parametric studies were performed with two-cell stage mouse embryos to determine the effect of concentration on the survival of the embryos. This animal model was chosen for parametric experiments because it proved extremely sensitive to the effect of the glycopeptides. While no survival of embryos was achieved without the glycopeptides (0%), very high survival of embryos was obtained with the glycopeptides (82.5%, in vitro development to the blastocyst stage). The details of the parametric studies are listed in Table 6. The experimental procedures for pig oocytes and pig embryos are found in Example 4 below, and for mouse embryos is found in Example 5.

Table 6 lists the results of the experiments of Examples 3 and 4 below, starting with the pig oocytes, followed by pig embryos and mouse embryos. Table 6 also shows the solutions in which the embryos and the oocytes were tested.

TABLE 6

EFFECT OF AFGP ON OOCYTE VIABILITY

Time of Exposure Soln (hr)	PBS	PBS + 0.1 mg/ml AFGP 1-8	PBS + 1 mg/ml AFGP 1-8	PBS + 40 mg/ml AFGP 1-8	PBS + 40 mg/ml AFGP 1-5	PBS + 40 mg/ml AFGP 7,8
4	6/48 (12.5%)	7/25 (24%)	19/27 (70%)	54/70 (77%)	12/43 (28%)	11/47 (23%)
4	9/48 (18.75%)	11/29 (37%)	20/27 (74%)	59/70 (84%)	21/43 (48%)	18/47 (38%)
24	0/17 (0%)	0/14 (0%)	6/14 (42%)	7/17 (41%)	0/14 (0%)	0/13 (0%)
24	0/17 (0%)	0/14 (0%)	9/14 (64%)	9/17 (53%)	0/14 1(0%)	0/13 (0%)

CRITERIA A = $\frac{\text{number of cells with electrical potential} > |u| - |v|}{\text{total number of cells}}$

CRITERIA B = $\frac{\text{number of with electrical potential} > |u| - |2v|}{\text{total number of cells}}$

The protocol to which the cells were exposed is the one described earlier in which the embryos and oocytes were introduced in various solutions with some of the embryos and oocytes exposed to rapid cooling while others which did not undergo cooling, kept as controls for the solution effect. The results are presented, for pig oocytes, as the ratio between the number of oocytes which reached the MI or MII stage after in vitro maturation, and the total number of oocytes exposed to the experimental protocol. For the pig embryos, it is the ratio between the number of embryos that reached the four-cell stage after in vitro development and the total number of embryos exposed to the experimental protocol. For the mouse embryos, it is the ratio between the number of embryos that reached the blastocyst stage over the total number of embryos exposed to the experimental protocol. The numbers in the bracket give the ratio expressed as percentage.

The experiments with pig oocytes, pig embryos, and mouse embryos exposed to the AVS solution, show that this solution does not have a damaging effect. However, when the embryos and the oocytes were cooled rapidly or vitrified to cryogenic temperatures in the AVS solution, not a single embryo or oocyte survived. These results demonstrate that the damage to these cells is a consequence of cooling and exposure to cryogenic temperatures. Microscopic examination revealed that a primary site of damage following rapid cooling in the AVS solution was the oolemma in the case of oocytes and the blastomer membrane for embryos which did not retain integrity as illustrated in the Figures, especially in Figs. 4B, 5A, and 6B. However, in the presence of the glycopeptides the cells that were rapidly frozen or vitrified retained viability as shown in Table 4.

In Figures 12 and 13 are shown the membrane potential for oocytes at 4 and 24 hr at 4°C. The dramatic retained membrane potential viability at concentrations of 1-40 mg/ml of AFGPs is found in Figure 13. Figures 12 and 13 values are mean ± one standard deviation. Each exp. group consists

of 5 oocytes and n represents the number of groups.

In particular, as described in experiment 3, the cell membrane was protected by the glycopeptides.

Bovine Oocyte Preservation

Past research on the properties of "antifreeze" proteins has focused on their ability to modify ice crystal structure on the mechanism by which this is achieved (Refs. 39,40,12,41). On the other hand, these results show that AFGP protects mammalian oocytes at cryogenic temperatures (-130°C) (Ref. 42) and at hypothermic temperatures (4°C) (Ref. 43). Porcine oocytes are normally completely destroyed by cryogenic temperatures (Refs. 42,22,44). However, when they were rapidly cooled to -130°C in a vitrifying solution containing AFGP, approximately 80% of them retain intact oolemmas and 25% undergo in vitro maturation (42). In follow-up experiments exposing porcine intact oocytes to 4°C for 24hr in the presence of AFGP, it was observed that 80% retained an intact oolemma and about 70% a normal membrane potential. In the absence of AFGP the integrity of the oolemma is completely destroyed (43).

The following (Example 9) was conducted to answer two general questions based on the foregoing results. Are the AFGP unique in their ability to protect cell membranes from hypothermic damage, or is this protection a general property of all known antifreeze protein classes? Does the membrane protection afforded by the antifreeze proteins improve the viability of cold-sensitive mammalian cells? To answer these questions, experiments were performed with immature bovine oocytes exposed to hypothermic temperature conditions.

Results and Discussion Regarding Bovine Oocytes

Approximately 1000 oocytes were individually studied in the experiments reported here. The results are summarized in Figures 14 and 15. Figure 14 shows the percentage of oocytes with an intact oolemma after hypothermic exposure as determined by three different tests, morphological examination, fluorescein diacetate (FDA) staining and trypan

blue (TB) exclusion. It is evident that only 10% to 25% of the control oocytes retained an intact oolemma following exposure to 4°C. Similar results were obtained for oocytes incubated at 4°C in PBS solution to which 0.1 M sucrose or 20% v/v fetal calf serum was added. In contrast, when the various AFPs were added to the incubation media, the integrity of the oolemma was retained in 50% to 75% of the oocytes, according to the morphological test used. The level of cell membrane protection from hypothermic exposure afforded by the different AFP was similarly high despite their different primary and secondary structures.

Evidence that the various AFP protected the viability of the oocytes is presented in Figure 15. While only 23.5% of the control oocytes exposed to hypothermic conditions underwent in vitro maturation, 64% to 75% of them matured when exposed to the same conditions in the presence of the various "antifreeze" proteins. This percentage maturation compares well with that observed for fresh oocytes (80%). Heyman et al. (45) reported a 32% maturation rate for bovine oocytes exposed to 0°C for only 30 min. These authors found that the addition of 0.1 M or 0.25 M trehalose, a membrane stabilizing compound, did not significantly increase the maturation rate. Similar negative results were found when 0.1 M sucrose or 20% v/v fetal calf serum was added to the PBS solution.

The results from the in vitro fertilization experiments illustrate clearly the biological significance of the protective effects of the thermal hysteresis protein ("antifreeze" protein). None of the control oocytes exposed to hypothermic temperatures in the absence of "antifreeze" proteins underwent in vitro fertilization, whereas 40-50% of the oocytes incubated in the presence of the antifreeze proteins could be fertilized. This rate of fertilization is comparable to what we normally obtain for fresh immature oocytes (60%).

These results clearly demonstrate that all of the different types of thermal hysteresis proteins ("antifreeze"

protein) currently identified in nature share the ability to protect cells and their membranes from damage during hypothermic exposure. It is evident that the hypothermic protection afforded by the antifreeze proteins extends to the functional viability of the cell. This observation further suggests that hypothermic protection may be a fundamental property and function of all of the "antifreeze" proteins in nature.

Our demonstration that the antifreeze glycoproteins (AFGP) enables cold-sensitive oocytes to maintain a normal membrane potential following exposure to hypothermic temperatures suggests that these proteins can prevent ion leakage. Recent experiments support this by demonstrating, using patch clamp techniques, that antifreeze proteins of different types do control and inhibit ion channels in mammalian cell membranes. These Examples demonstrate that all three of the known antifreeze protein types can protect the integrity of cold-sensitive bovine oocytes to the point where they remain fully functional. Taken together these results support the present invention, that by reducing the leakiness of the cell membranes, cold-sensitive cells become cold tolerant. The practical value of the finding that antifreeze proteins can render cold-sensitive mammalian cells cold-resistive is of importance to the short term preservation of cold-sensitive mammalian cells, particularly in the viable preservation and storage of tissue or organs for transplantation.

These thermal hysteresis proteins are therefore useful in calcium entry therapy for diseases.

UTILITY

It is apparent from the disclosure herein that the thermal hysteresis protein (AFP and AFPG) aqueous composition of the present invention is useful in cell preservation, membrane preservation, tissue preservation or organ preservation.

In general, the antifreeze proteins have the property that they noncolligatively lower the apparent freezing point

of aqueous solution resulting in a freezing temperature that is lower than the melting temperature. They also have the general property that they inhibit or restrict growth on different facets of ice crystals while allowing the growth along the c-axis. Until now it was not know that these proteins can be also use to interact with other proteins, and, in particular, protein on the surface of cell membrane and to protect the structural integrity of the membrane and stop leakage through the membrane and block ion channels. This is the first time that these properties and its applications are observed and are described as part of this invention.

EFFECT OF THE AFGP'S ON THE CELL MEMBRANE

Initially, the effect of the antifreeze proteins on modification of ice crystal growth focused on the use of this property in preservation of cells, tissue, organs and whole animals at temperatures below freezing. However, in studies described above and experiments such as Examples 1 and 4 (also Table 4), in which the morphology of cell membranes was evaluated, it appeared consistently that the antifreeze proteins provide complete protection to the morphology of the membrane and its structural integrity. Therefore, a procedure was developed to determine if the antifreeze proteins protect by interacting directly with cell membranes, and contacting the protein directly to cell membranes.

Pig oocytes were initially chosen as the experimental model in this study because these oocytes are temperature sensitive and cannot survive exposure to hypothermic temperatures as high as 10°C, i.e., temperatures that are higher than the phase transition temperature. Therefore, an experiment was designed in which the effect of the AFGP's on the cell was studied at temperatures higher than the phase transition temperature, but lower than the normal body temperatures. If a protective effect of the AFGP's is found, it is probably not directly related to the ability of the compound to modify ice crystal morphology or inhibit of

ice crystal formation.

The oocytes were introduced in different solutions of standard buffer PBS solution with antifreeze glycoproteins (Fractions 1-5, Table 1) from fish of the family Notothenidae. They were kept in a constant temperature environment for various periods of time and then the membrane potential was measured. The structural integrity was also determined by microscope evaluation.

To establish a criteria for an intact oolema, preliminary experiments were performed for each batch of oocytes in which the membrane potential of the fresh oocytes was measured at 22°C. The mean value of the electrical potential, u, and the standard deviation, v, were calculated for each batch. The mean and the standard deviation were measured in fresh oocytes in a buffer solution and in a buffer solution with 40 mg/ml antifreeze glycopeptides Fractions 1-8 (Table 1) as obtained from A. DeVries, supra.

Table 6 above summarizes the results derived from measuring the resting potential across the oolemma. Table 4 gives the ratio between the number of oocytes considered to have an intact oolema relative to the number of oocytes used for each experimental condition, (the number in the brackets is the ratio in percentage,) for different concentrations of the AFGP's and different times at 40°C.

The comparison shows that the glycopeptides have little effect on the resting potential of each oocyte. To determine the integrity of the oolema, two statistical criteria were established, one less stringent than the other. The oolema in an oocyte was considered to be intact if the absolute value of the measured resting potential difference, was higher than the absolute value of either $|u|-|v|$ or $|u|-|2v|$.

The results from evaluating the structural integrity of the oolema are consistent with the electrical potential measurement and are illustrated by Figures 7A, 7B, and 7C. The results clearly show that the membrane is preserved morphologically intact in the presence of the glycopeptides.

Furthermore, ion leakage that is probably the most prevalent cause of damage during hypothermic exposure is significantly inhibited in the presence of the antifreeze proteins. This implies that the antifreeze proteins have the ability to protect cell membranes at hypothermic temperatures and to block ion channels. The evidence of the use of this new discovery in hypothermic preservation of cells and organs are given below in Examples 6 and 7, respectively.

It is emphasized that prior to this research it was not known that antifreeze proteins have the useful properties of preserving cell membranes and blocking ion channels.

WHOLE ORGAN PRESERVATION

Cryopreservation of a whole organ, e.g. liver from a mammal, such as a rat, is described in Examples 2, 7 and 7A below. The organ is surgically removed, held in a preservation solution at 20-37°C, preferably 24°C. A major blood vessel is cannulated. The well-known Langendorf perfusion system (with a first bottle containing, for example, Krebs solution and antifreeze polypeptides in a 1mg/ml to 100 mg/ml) is used. See, for example, D.E. Pegg et al. (1986), Cryobiology, Vol. 23, pp. 150-160.

A second bottle of solution contains a physiologically compatible saline solution and appropriate quantities of glycerol, dimethyl sulfoxide, ethylene glycol, polyvinyl pyrrolidone, glucose etc. or mixtures of these substances which are known as protectants for cells of biological origin.

These two bottles of solutions are connected to a mixing valve having known adjustable flow rates (e.g. 0.1 to 10 ml/min, preferably about 5 ml/min) and a computer to accurately vary the flow rate and mixing of each bottle's contents immediately prior to perfusion. The perfusion using the solutions of bottles 1 and 2 is well known in the art as described by D.E. Pegg et al. (1988) above for kidney and G.N. Alink et al. (1976), Cryobiology, Vol. 13, pp. 295-304; (1977) Cryobiology, Vol. 14, pp. 409-417 and 399-408; and (1978) Cryobiology, Vol. 15, pp. 44-58, and K.E.F. Hobbs

et al. (1969), Cryobiology, Vol. 6, pp. 239-245 for heart. The Krebs solution is perfused through the organ held at about 20-37°C at a rate of about 4 ml/min.

The mixing switch provides intermediate amounts of Krebs solution and glycerol solution in pulses of time lengths controlled by the computer, for example, 0.01 sec. to 0.1 sec. The two solutions mix in the delivery tube or in a special mixing chamber.

The antifreeze protein/Krebs solution is initially adjusted so that at the end of the perfusion process a concentration of between 1mg/ml to 40 mg/ml is achieved in blood vessel space in the tissue. The majority of the AFPs are found within the vascular space (bed) of the organ (not within the cells of the liver or the blood vessels). The AFPs ("AFGP's") are usually too high in molecular weight to significantly penetrate the cell membrane. The organ, e.g. liver, is next placed in a cooling stage as described in U.S. Patent 4,531,373, and the temperature of the whole perfused organ is then cooled at a rate of 1°C per minute to -32°C or to -70° or until -150°C is reached. The organ is then cooled as needed using a liquid nitrogen to -196°C or in liquid helium to 4K and held at this temperature for an indefinite time (e.g. 72 hr). The frozen organ is then carefully thawed by immersion in a cold or warm liquid, e.g. water or saline, at a rate of between about 0.1 to 10°C per min. (preferably about 1°C per min.) using known techniques up to 37°C maximum. Alternatively, carefully controlled microwave heating is used to thaw the perfused organ, e.g. liver. When the thawed organ (liver) reaches about 0°C, the nutrient solution of Krebs is perfused through the large cannulated blood vessel. When warmed to about 20 to 37°C, preferably 37°C, the thawed organ recovers not only cell function, but also organ function. Preserved tissue samples are taken as needed.

A systematic study of the effects of the AFGP on rat liver cold-storage was done to compare control storage solutions and solutions containing the AFGP (see Example

7A). The results obtained are compared for three different storage periods, 6, 12 and 24 hr. The functional tests include the production of bile and enzymatic activities of lactic dehydrogenase (LDH). The Krebs solution is selected as the control solution. The reason for this selection is to separate the protective qualities of other storage solutions from the effects of the AFGP.

Useful mammalian organs include liver, kidney, heart, brain, lung, pancreas, spleen, ovary, stomach and the like. The organ of a mammal, such as a human being, is preferred.

PRESERVATION OF CELLS AND TISSUE AT TEMPERATURES BELOW FREEZING

The cryopreservation is demonstrated in cells, e.g. human ooytes, pig oocytes, embryos, human or leucocytes, platelets, e.g. pancreatic islets, hepatocyles, corneas, skin. See examples 4 and 5. Various cryoprotective agents such as glycerol, propylene glycol are introduced in the cell together with the antifreeze proteins essentially as described in Examples 3, 4 and 5. The different solutions of cryoprotective agents are chosen to either produce freezing or vitrification such as 5M propylene glycol. The cells or tissues are then cooled rapidly to either produce freezing or vitrification with cooling rates of e.g. 1750 °C/min or as high as required to temperatures of -130°C to -180°C, or to 4K and held at that temperatures for an indefinite period. The cells or tissue are then carefully thawed. Cell function and tissue function is recovered.

PRESERVATION OF ORGANS BY RAPID FREEZING VITRIFICATION

The procedure is the same as described in the whole organ preservation section except that the concentration of the cryoprotectant is taken to a high level, such as 5M propyleneglycol, and the cooling rates are high enough, such as 1,750°C/min, to produce either rapid freezing or vitrification as desired as opposed to slow freezing in the earlier application. The use of antifreeze proteins is essential for the successful preservation of organ tissue by vitrification.

HYPOTHERMIC PRESERVATION OF CELLS

The procedure of Example 6 is followed except that liver cells are first contacted with aqueous AFGP solution. These cells survive the cooling and are viable upon careful warming to physiological temperatures.

HYPOTHERMIC PRESERVATION OF ORGANS IN COLD STORAGE

The procedure of Example 6 is followed except that an organ, e.g., liver or heart is contacted with the aqueous AFGP solution. This organ survives the cooling and is viable upon careful warming to physiological temperatures.

HYPOTERMIC PRESERVATION OF ORGAN BY CONTINUOUS PERFUSION

Example 7 is repeated except that the blood containing the antifreeze glycoprotein is continuously perfused through the organ.

HYPOTHERMIC PRESERVATION OF CELLS, TISSUE, ORGANS, MAMMALS

Example 6 is repeated with the exception that antifreeze proteins are brought in contact with cells, tissue, organs, mammals where it is desired to protect them from hyperthermic damage.

PRESERVATION OF CELLS, TISSUE (SKIN), ORGANS, MAMMALS FROM A CHEMICAL ENVIRONMENT THAT IS NOT OPTIMAL

Example 6 conditions are repeated with the exception that a non physiological chemical environment such as high carbon dioxide is present.

PRESERVATION OF CELL MEMBRANES

Cell membranes are brought into contact with physiologically capatible solutions with antifreeze proteins.

BLOCKING ION CHANNELS

Cell membranes are brought into contact with physiologically compatible solutions with antifreeze proteins. In channels, for example, sodium and postassium are found to be substantially blocked.

ATTACHMENT THROUGH ANTIFREEZE PROTEINS

Various macromolecules are artificially attached to

antifreeze protein and than introduced in cell suspension, tissue, organs, or whole mammals. The antifreeze protein attach then to cell membranes and thereby bring molecules in the vicinity of the cell membrane.

The preservation of these mammalian organ tissues, etc. is also applicable to the preservation of organs, tissue, etc. in a human being.

Ion Current Determination in Pig Granulosa Cells using Thermal Hysteresis Proteins

Observed hypothermic protection of cells of cell membranes conferred by the thermal hysteresis protein (e.g.,) "antifreeze" proteins) is associated with their ability to inhibit ion leakage. The effects of "antifreeze" proteins from the winter flounder (Pseudopleuronectes americanus), one of the better characterized "antifreeze" proteins was evaluated on known and well characterized ion currents. Pig granulosa cells were used to characterize the potassium and calcium currents using the patch clamp technique in the whole cell configuration (Ref. 46).

The "antifreeze" polypeptides (AFP), from the winter founder (Pseudopleuronectes americanus) consist of a family of seven independently active components containing eight amino acids of which alanine forms about 60 mol. %. Most of the remaining residues are polar: aspartic acid, glutamic acid, lysine, serine and thereonine (Ananthanarayanan, 1989). The compounds range in molecular weight from 3300 Da to 4500 Da. The two major AFP (3300 Da) found in the blood plasma have been sequenced (Picket et al. 1984) and one of them crystalized (Ref. 4). They are amphiphilic alpha-helices in which the majority of the hydrophylic amino acid side chains project along the length of one side of the helix while the opposite side, is predominantly hydrophobic (Ala). Studies on their antifreeze properties show that they lower the freezing point of a solution in a non-colligative way and have no effect on the melting point of the formed ice. This difference between the freezing and melting points of the AFP solution is thermal hysteresis,